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Highly bright avidin-based affinity probes carrying multiple lanthanide chelates

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ABSTRACT

Lanthanide ion luminescence has a long lifetime enabling highly sensitive detection in time-gated mode. The sensitivity can be further increased by using multiple luminescent labels attached to a carrier molecule, which can be conjugated to an object of interest. We found that up to 30 lanthanide chelates can be attached to avidin creating highly bright constructs. These constructs with Eu^{3+} chelates display synergistic effect that enhance the brightness of heavily modified samples, while the opposite effect was observed for Tb^{3+} chelates thereby significantly reducing their light emission. This undesirable quenching of Tb^{3+} luminophores was completely suppressed by the introduction of an aromatic spacer between the chelate and the protein attachment site. The estimated detection limit for the conjugates is in the 10^{-14}–10^{-15} M range. We demonstrated a high sensitivity for the new probes by using them to label living cells of bacterial and mammalian origin.

1. Introduction

Long lifetime of lanthanide luminescence allows its highly sensitive detection in time-gated mode [1–9], making luminescent probes an attractive alternative to radioisotopes. To compensate for the low inherent absorbance of lanthanide ions, the luminescent probes contain an antenna fluorophore, which absorbs the light and transfers the energy to a tethered Ln^{3+} ion that finally emits the light [3 and references therein]. One of the ways to significantly increase the detection sensitivity of light-emitting probes is to bundle them onto a carrier molecule, which then can be attached to an object of interest [10,11]. With conventional fluorophores this approach is complicated due to self-quenching, which is facilitated by the fluorescence resonance energy transfer (FRET) from an excited to a nearby non-excited dye molecule that efficiently absorbs the energy [10,11]. The degree of quenching is highly dependent on the spectral overlap between the excitation (absorption) and emission of a particular fluorophore. The majority of conventional fluorophores have a small (10–30 nm) Stokes shift (the spectral separation between the emission and absorption maxima) causing a significant spectral overlap. High molar extinction of the common fluorescent dyes also contributes to quenching. On the contrary, lanthanide luminescent probes possess an extremely large Stokes shift (150–250 nm), which prevents efficient energy transfer between the excited and non-excited fluorophore molecules [12]. Previously, this approach was explored on streptavidin with Eu^{3+} chelate [12]. Parent protein, avidin possesses 32 lysine residues at which luminescent labels can be attached, which makes it a superior scaffold for multiple label attachment comparing to streptavidin (which has 12 lysine residues). In the present study, we obtained avidin conjugates with a new generation of high-quantum-yield lanthanide chelates of Eu^{3+} and Tb^{3+} containing cs124 and cs124-CF_3 antennae-fluorophores (Fig. 1) synthesized by us in the course of current and previous studies [13]. We find that unlike typical fluorophore BODIPY, the light emission efficiency of the Eu^{3+} probes was not affected by self-quenching. In fact, the cumulative luminescence of the conjugate as a function of the number of the attached residues displayed a super-linear behavior, suggesting synergistic effect [12]. We found that this effect was due to the enhanced antenna-to-lanthanide energy transfer. We tested the same approach with Tb^{3+}-based luminescent probes, which possess higher quantum yield compared to the cs124 Eu^{3+} chelates. Significant self-quenching was observed when these multiple Tb^{3+} probes were attached to avidin. However, introduction of a biphenyl spacer between the chelate and the crosslinking group completely suppressed the quenching, yielding highly bright conjugates. The obtained luminescent avidin constructs were used for labeling bacterial and mammalian cells giving highly contrast images in time-resolved detection mode.

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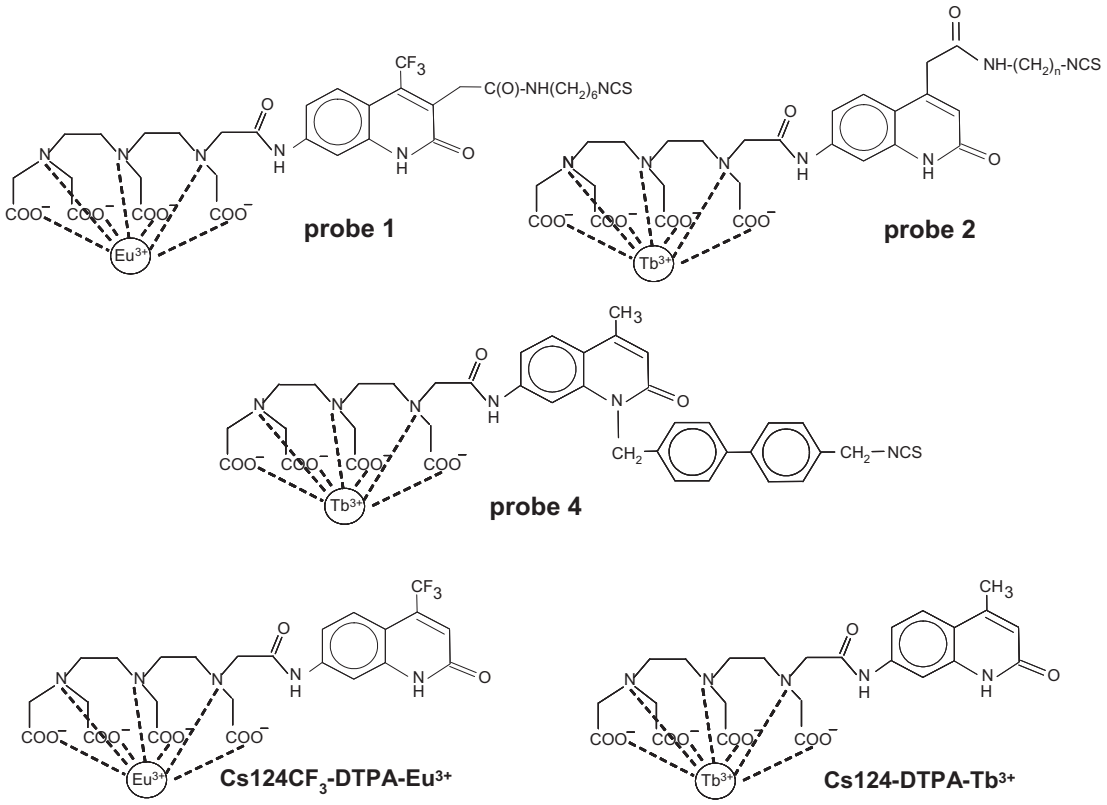

Fig. 1. The structure of the luminescent probes **1**, **2**, **4** and reference non-reactive compounds.

These new probes can find a broad range of applications in the biological and biomedical fields that rely on high detection sensitivity.

2. Materials and methods

The following reagents were purchased from Sigma Aldrich: Avidin, diethylenetriaminepentaacetic acid dianhydride (DTPA), triethylamine; butylamine; 1,3-phenylenediamine; ethyl 4,4,4-trifluoroacetoacetate; ethylacetoacetate, 1,3-dicyclohexylcarbodiimide (DCC), ethylenedianime; methylbromacetate; anhydrous dimethylformamide and dimethylsulfoxide; 1-butanol, ethylacetate, chloroform; acetonitrile; ethanol; sodium and potassium hydroxide; $TbCl_3$ and $EuCl_3$; silica gel TLC plates on aluminum foil (200 μm layer thick with a fluorescent indicator). Distilled and deionized water (18 MΩ cm^{-1}) was used. All experiments including lanthanide complexes preparation and using thereof were performed either in glassware washed with mixed acid solution and rinsed with metal-free water, or in metal-free plasticware purchased from Biorad. All chemicals were the purest grade available. Probes I and II were synthesized as previously described [13]. Biotinylated oligonucleotide containing BHQ had a structure: 5′NH_2 – ACCTGGTGCCTCGTCGCCGCAGCTCAGG dT (BHQ2) TT-3′ – biotin. NHS-$dPEG_{12}$-biotin was purchased from Quanta Biodesign.

2.1. Synthesis

2.1.1. Probe 4 (Fig. 1)

*2.1.1.1. Product **I**.* To a solution of 106 mg (0.6 mmol) of cs124 in 0.8 ml of DMF 72 μl of 10 M NaOH was added followed by rigorous agitation until the water phase disappeared. This solution was mixed with a 300 mg 4,4′-bis (chloromethyl) biphenyl dissolved in 2 ml of DMF. After 20 min incubation at room temperature the TLC analysis in hexane–acetone (1:1) revealed the formation of a single reaction product. The mixture was supplemented with 100 mg of lithium azide and heated for 20 min at 50 °C followed by precipitation with 20 ml of water. The residue was collected by centrifugation, washed with water and dissolved in 20 ml of hot acetonitrile. The acetonitrile was removed by evaporation under reduced pressure and the residue was washed a few times with hot hexane and subjected to silica gel chromatography in hexane–acetone (1:1) developing system. Yield-120 mg. ^{1}H NMR in DMSO:7.65 (dd, 4H, o,o′biphenyl H, J_1 = 11.1, J_2 = 8.4), 7.45 (dd overlapped, 1H, 5H), 7.45 (dd, 2H, biphenyl m-H, J_1 = 8.25, J_2 = 5.1), 7.25 (d, 2H, biphenyl-m′- H, J = 8.1), 6.49 (d, 1H, 6H), 6.44 (dd, 1H, 3H, J = 1.8), 6.21 (s, 1H, 8H), 5.8 (s, 2H, 7 amino), 5.38(s, 2H, N-CH_2), 4.4 (s, 2H, -CH_2-N_3), 2.36 (d, 3H, 4-methyl, J = 0.9).

*2.1.1.2. Product **II**.* Solution of 68 mg of product **I** in 0.5 ml of DMF was supplemented with 1.5 M excess of triphenylphosphine, incubated for 1 h at 50 °C and 0.13 ml of 25% aqueous ammonium hydroxide was added. The mixture was incubated for 1 h at 50 °C and left for 20 min at −20 °C. The precipitate was collected by centrifugation, washed by ether and dried *in vacuo* affording 36 mg of product **II**.

*2.1.1.3. Product **III**.* The solution of 30 mg of product **II** in 0.5 ml of DMSO was titrated with thiocarbonyldiimidazole dissolved in 0.1 ml of chloroform. Addition was continued until the subsequent portion of C(S) Im_2 stopped decolorizing. The reaction mixture was analyzed by TLC in hexane–acetone (1:1) developing system revealing nearly complete conversion of the original cs124 derivative. Small excess of C(S) Im_2 was required to complete the reaction. The mixture was supplemented with 5 μl of TFA and left for 1 h at 45 °C. The reaction was monitored by TLC. The product was precipitated by water (13 ml), collected by centrifugation

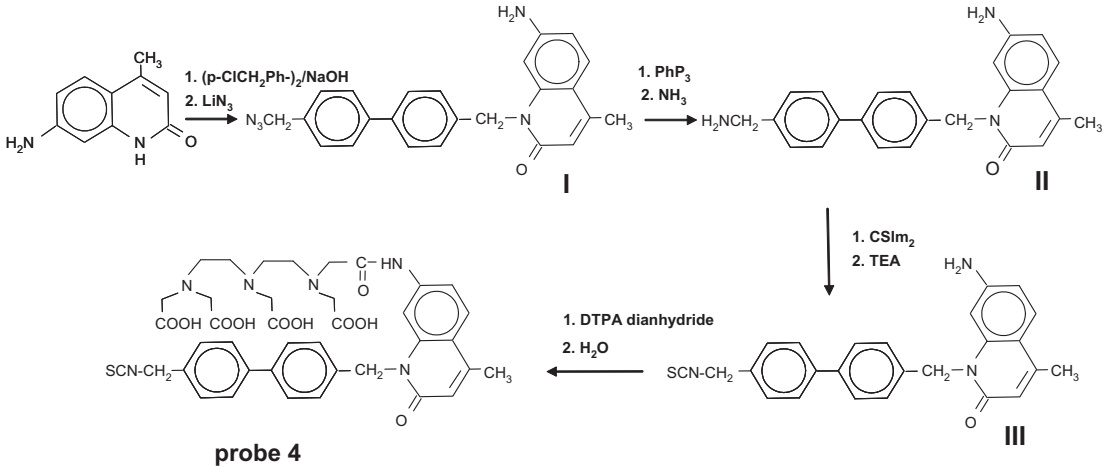

Fig. 2. Synthetic route for probe **4**.

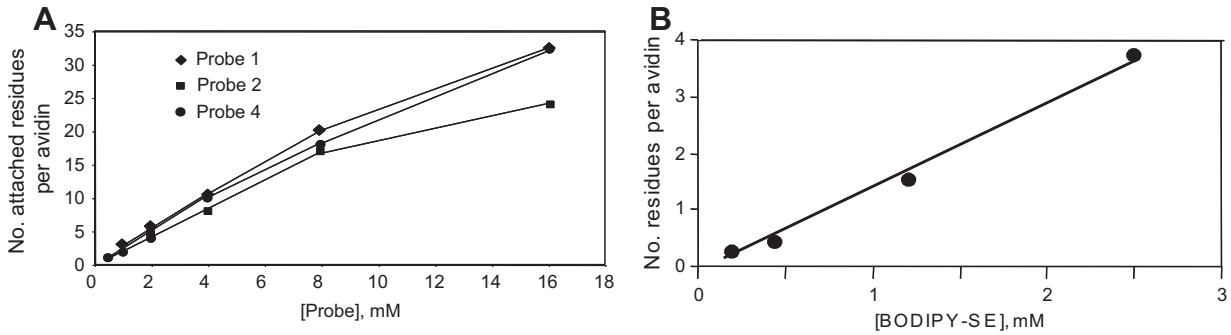

Fig. 3. Dependence of the number of the attached probes **1**, **2**, and **4** (A) and BODIPY residues (B) per avidin on the reactive fluorophores concentration in the reaction mixture.

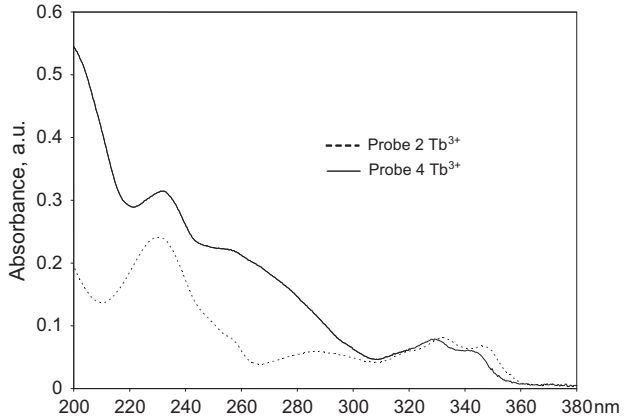

Fig. 4. UV absorption spectra for Tb chelates of probe **4** and probe **2**.

and washed by water two more times. Most of the remaining residue was dissolved in 10 ml of acetonitrile and the remaining material was removed by centrifugation. Acetonitrile solution was evaporated to dryness *in vacuo* affording 20 mg of product **III**. ^{1}H NMR in DMSO:7.66 (m, 4H, o,o′biphenyl H), 7.48 (dd overlapped, 1H, 5H, J = 2.1), 7.45 (d, 2H, biphenyl m-H, J = 8.1), 7.3 (d, 2H, biphenyl-m′- H, J = 8.4), 6.7 (s, 1H, 8H), 6.62 (dd, 1H, 6H, J_1 = 9.0, J_2 = 1.8), 6.55 (d, 1H, 3H, J = 1.8), 6.25 (s, 2H, 7 amino), 5.5 (s, 2H, -CH_2-NCS), 4.48 (s, 2H, N-CH_2).

2.1.1.4. Probe 4. Solution of 35 mg (0.1 mmol) of DTPA dianhydride in 0.3 ml of DMSO obtained under heating to 60–80 °C was cooled down to room temperature and added to 20 mg (0.048 mmol) of compound **III**. The reaction was carried on for 15 min at 20 °C. The mixture was supplemented with 4 ml of water, left for 20 min at room temperature and pH was adjusted to 5.0 by LiOH. The product was purified by preparative C-18 HPLC column (20 × 250 mm) using linear gradient (0.5l) of acetonitrile in water (0–70%). The elution rate was 2 ml/min. The fractions containing desired product were combined and supplemented with one equivalent of a lanthanide salt. The resulting solutions were concentrated *in vacuo* by co-evaporation with acetonitrile under gentle heating (25–30 °C) to final concentration 20 mM.

2.2. Modification of avidin with light-emitting probes

The reaction cocktails (10–16 μl) were composed by mixing of 7 μl of avidin (20 mg/ml), 1 μl of 1 M sodium borate buffer pH 10.0, and 1–8 μl of a reactive light-emitting probe at concentrations specified in figure legends. After incubation for 4 h at 56 °C the mixtures were diluted to 100 μl by water and subjected to size-exclusion chromatography on Sephadex G-50 "medium" in 10 mM Hepes-HCl buffer pH 8.0 containing 50 mM NaCl. The fractions corresponding to modified avidin were collected by visual detection using UV monitor (365 nm light).

2.3. Preparation of Escherichia coli cells labeled with modified avidin

LB broth (100 ml) was inoculated with suspension of 10 μl of *E. coli* cells (RL721 strain) and incubated in a 500 ml Erlenmeyer flask

Table 1
Fluorescence of avidin conjugates, non-attached probes, and reference compounds in water and deuterium oxide solutions.

Probe	No. residues per avidin	Fluorescence (per 10 nM avidin) in H_2O	Fluorescence (per 10 nM avidin) in D_2O	Specific (calculated for fluorescence 10 nM fluorophore)	
				in H_2O	in D_2O
1	31	67,500	210,000	2250	7000
2	24	75,000	100,000	3100	4200
4	30	377,000	520,000	12,600	17,300
2 (non-attached)	N/A	N/A	N/A	34,500	42,000
4 (non-attached)	N/A	N/A	N/A	30,700	37,000
1 (non-attached)	N/A	N/A	N/A	2300	7500
Reference compounds (see Fig. 1)					
Csl24-DTPA-Tb^{3+}	N/A	N/A	N/A	40,000	65,000
CS124CF -DTPA-Eu^{3+}	N/A	N/A	N/A	2500	10,000

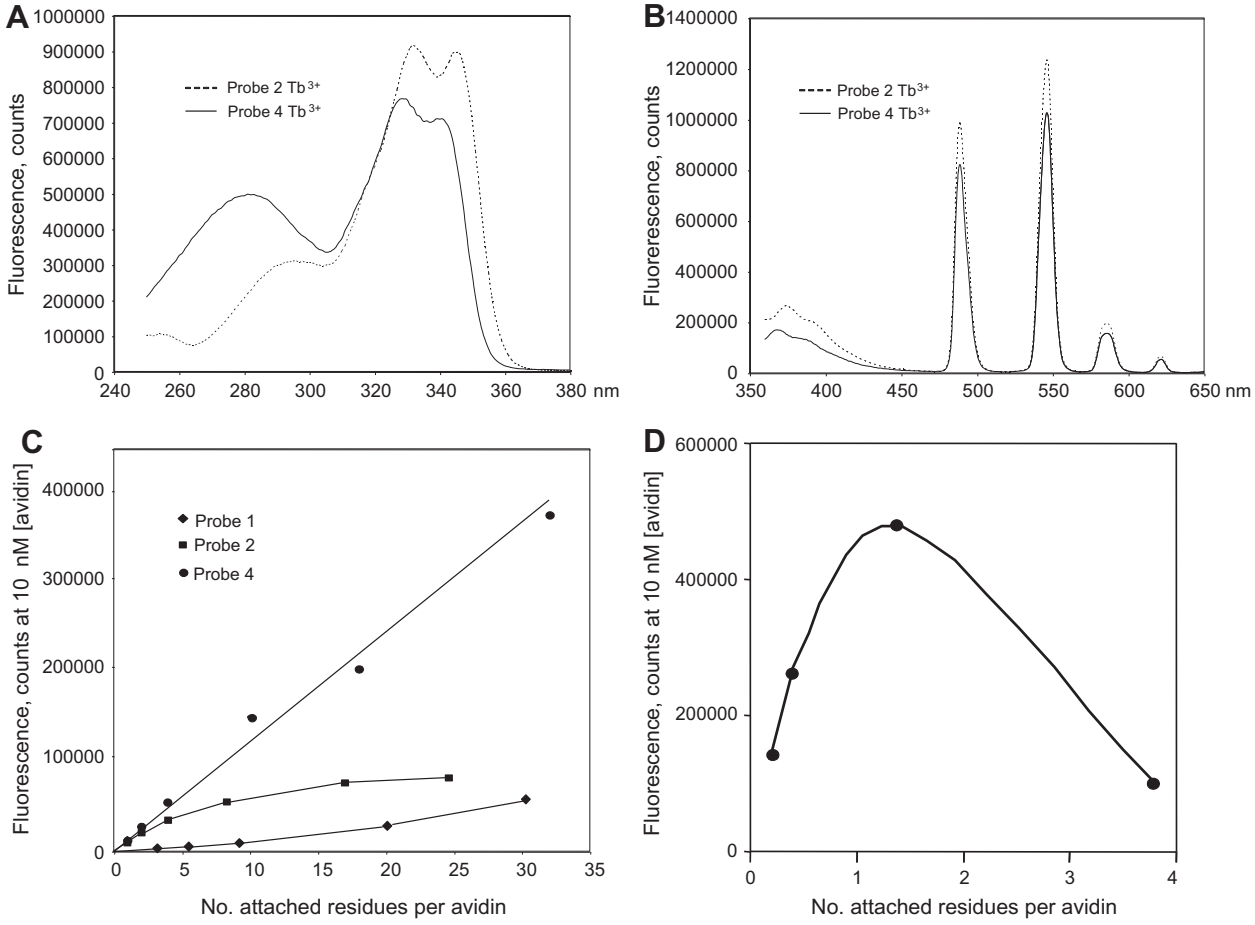

Fig. 5. Light emitting properties for lanthanide chalates and for avidin modified with multiple labels. Excitation (A) and emission (B) spectra for probes **4** and **2**. Excitation spectra were recorded at emission wavelength 490 nm. Emission spectra were recorded at excitation wavelength 329 nm (for probe **4**), and 332 nm (for probe **2**). C, D, dependence of the cumulative fluorescence of the avidin modified with probes **1**, **2**, and **4** (C) and BODIPY (D) on the number of the attached residues.

overnight at 37 °C. The cells were harvested by centrifugation (4000 rpm, 5 min), washed with PBS and re-suspended in the same buffer containing 50% glycerol at a final density of 32 mg ml^{-1}. Thirty microliters of this suspension containing ca. 1 mg of cells was washed 3 times with 1 ml of 0.1 M sodium borate buffer, pH 8.5, and each time collected by centrifugation. After the last wash, the cells were suspended in 50 μl of the same buffer and 4 μl of 100 mM DMSO solution of NHS-$dPEG_{12}$-biotin was added. After incubation at room temperature for 30 min the cells were washed 4 times with 500 μl of PBS. After the final wash, cells were suspended in 15 μl of PBS buffer and supplemented with 15 μl of

Table 2
Antenna-to-Eu^{3+} emission ratio for probe 1 attached to avidin in H_2O at pH 8 for 10 nM avidin.

No. of attached residues per avidin	Eu^{3+} emission (counts)	Antenna emission (counts)	Eu^{3+}/antenna emission ratio
3	3000	3760	0.79
6	7100	5000	1.42
20	42,000	21,700	2.0
31	67,500	26,000	2.6
10 nMPrIEu^{3+} (non-attached)	2300	2300	1.0

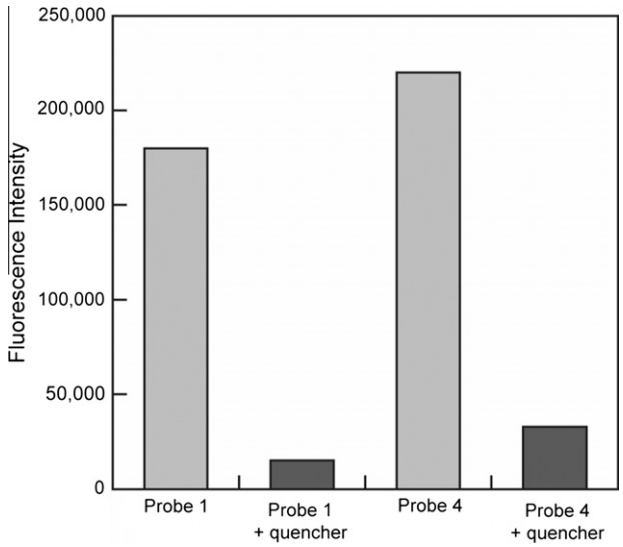

Fig. 6. Light emission spectra of luminescent avidin conjugates in the absence (grey bars) and in the presence (black bars) of biotinylated DNA oligo carrying a Black Hole Quencher.

5 μM avidin modified with one of the lanthanide labels [AV – Probe **4** -Tb^{3+} (n = 15) and AV – Probe **1**-Eu^{3+} (n = 19)]. After 25 min of the incubation at room temperature cells were washed by PBS (4 × 500 μl) and suspended in 100 μl of the same buffer.

2.4. Preparation of Chinese hamster ovarian cells (CHO) labeled with modified avidin

CHO cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 200 mM L-glutamine and 100 g/ml penicillin/streptomycin solution. Once the cells reach 80–90% confluency, they were trypsinized and collected by centrifugation (1000 rpm for 5 min), washed with 0.1 M Na-borate buffer pH 8.5 (3 × 0.5 ml) and spun down at 3000 rpm for 30 s. The cells were suspended in 500 μl of sodium borate buffer, divided into 5 equivalent volumes 100 μl each, and supplemented with various volumes (0.5 μl, 1 μl, 2 μl, and 4 μl) DMSO solution of 100 mM NHS-$dPEG_{12}$-biotin. 100 μl of the suspension was kept for auto fluorescence reference. The cells in each tube were washed with 150 μl of PBS buffer three times, suspended in 20 μl of PBS and supplemented with 15 μl of 5 μM AV – Probe **1**-Eu^{3+}. After incubation at room temperature for 20 min, the cells were washed with 100 μl of PBS buffer and suspended in 50 μl of the same buffer.

2.5. Preparation of the cells for microscopic detection

One microliter of Poly-L-lysine was spread onto a fused silica microscope substrate into an area of 0.3 cm^2 and removed. One microliter of the cell suspension of labeled cells (*E. coli* or CHO cells) containing 10^9–10^{10} cells cm^{-3} in PBS buffer was spread into the same area and left to air dry for 15 min.

2.6. Physical methods

Excitation and emission fluorescence spectra in the continuous excitation mode were recorded using QuantaMaster 1 (Photon Technology International) digital fluorometer at ambient temperature. Time-resolved and gated luminescence measurements were performed using the previously described home-built experimental set-up [13]. A Hacker Instruments Zetopan microscope was equipped with an ICCD Camera (PI-MAX, Princeton Instruments). In the experiments, the images were taken in luminescence light using evanescent wave excitation at 351 nm as well as in scattered light using standard top illumination by xenon lamp. In the evanescent excitation, a right angle fused silica prism was illuminated with laser light (351 nm) from a XeF laser (OPTEX, Lambda Physik). The sample was located on the hypotenuse side of the prism positioned horizontally. Images taken in scattered light from a xenon lamp were taken before and after the luminescent images collected in the photon counting mode. Online thresholding mode was used to discriminate photon pulses from the readout noise as well as the "cosmic events". The 1024 × 1024 camera pixels were 8 × 8 binned resulting in 128 × 128 $pixel^2$ images. The microscope used an objective with the magnification of ×56 and the numerical aperture of 0.90. Combined with the intermediate "ocular" lens with the magnification of ×10 it provided the field of view of 14 × 14 μm^2. In some experiments, an ×5 intermediate "ocular" lens was used resulting in the 28 × 28 μm^2 field of view.

2.6.1. Microscopic detection of living cells utilizing avidin labeled with multiple probes using Total Internal Reflection Fluorescence Microscopy (TIRFM)

The cells labeled with avidin carrying multiple probes (Probe **1**-Eu^{3+} and Probe **4**-Tb^{3+}) were placed on the hypotenuse side of the prism mounted at the microscope base. The excitation of probes occurred in the evanescent wave by laser light totally internally reflected from the hypotenuse side inside the prism. The probes with

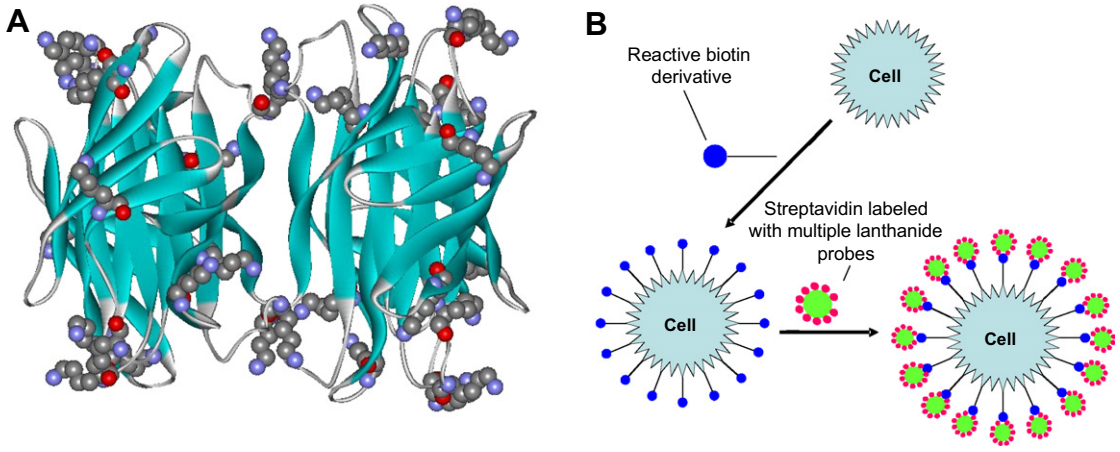

Fig. 7. The scheme for luminescent labeling of living cells. (A) X-ray structure of avidin displaying lysine residues to which reactive light-emitting probes can be attached. (B) The strategy for cell labeling. The cells are treated with reactive biotin derivative, followed by attachment of the luminescent labeled avidin conjugates to biotinylated cells.

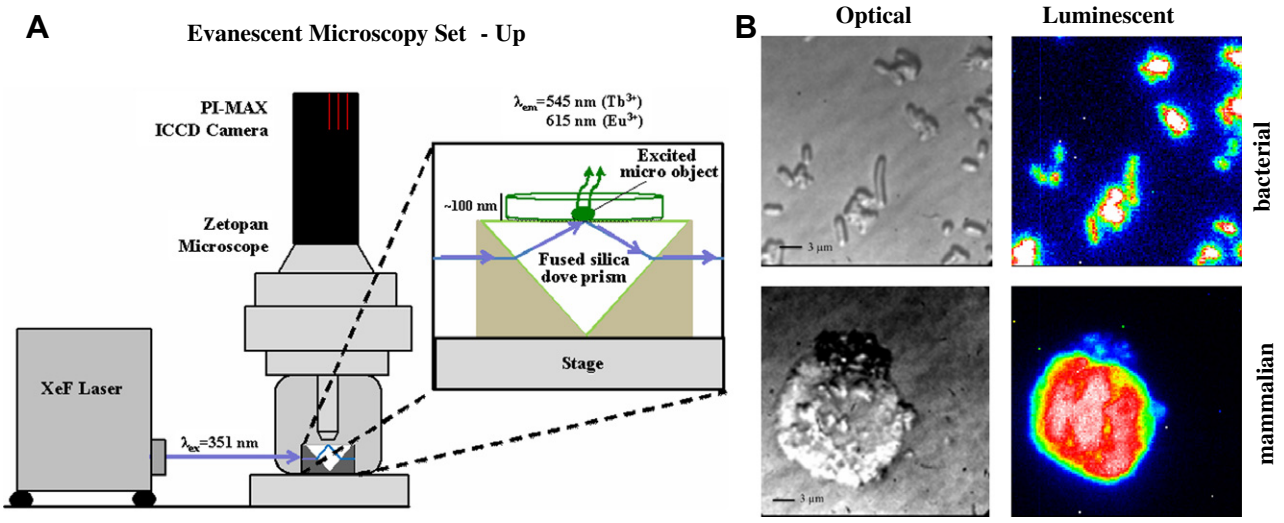

Fig. 8. Imaging of the labeled cells using TIRF microscopy. (A) The scheme of the device for time gated imaging. (B) Optical (left) and luminescent (right) images of *E. coli* (top) and Chinese hamster ovarian cells (bottom).

Eu^{3+} have emission lifetime of ca. 0.5 ms, while the probes with Tb^{3+} have emission lifetime of ca. 1.5 ms. Therefore, for samples labeled with Eu^{3+} probes we used a gate width of 1 ms and the gate delay of 50 μs and for samples labeled with Tb^{3+} probes 2 ms gate width and 100 μs gate delay were used. Images in luminescence light were acquired in the photon counting mode; typically 1000 pulses were accumulated (at the repetition rate of 2 Hz). The number of probes per cell was calculated based on the total photon count with the subtraction of the background count.

The calibration of the set-up was performed by collection of luminescence light from a thin layer of the probes solution excited directly by the laser beam at the right angle from the bottom of a thin fused silica substrate. The microscope field of view in these experiments was $14 \times 14\ \mu m^2$. To achieve homogeneity of the excitation beam, the beam was passed through a $0.32\ cm^2$ diaphragm. The pulse energy was measured after the diaphragm ($0.32\ mJ\ pulse^{-1}$). This allowed a reliable determination of the laser light fluence. Measured volume of the probes solutions (1.12 mM Probe **1**-Eu^{3+} or 0.107 mM Probe **4**-Tb^{3+}) in glycerol was placed on the top of the substrate and spread upon the surface with a cover slip (the spot area of $3.80\ cm^2$ and the thickness of the layer of 2.63 μm). The luminescence light intensity was calculated based on the photon fluence, the absorption cross-sections of the probes at 351 nm ($2.1 \times 10^{-17}\ cm^2\ molecule^{-1}$ and $3.6 \times 10^{-17}\ cm^2\ molecule^{-1}$ for probes Eu^{3+} and Tb^{3+}respectively), the luminescence quantum yield (0.167 for Eu^{3+} [14], and ca. 0.45 for Tb^{3+} probe), and the total number of probes in the field-of-view area. This was compared with the total number of photons counted in the image. This procedure allowed determination of the calibration coefficients, which lump sum the solid angle of light collection of the objective lens, the microscope throughput coefficient, the photocathode quantum efficiency, as well as the photon counting efficiency. The average number of the probes per externally labeled *E. coli* cells determined in this way was 2.1×10^5 and 2.9×10^5 for Eu^{3+} and Tb^{3+} probes, respectively.

Externally labeled CHO cells were prepared in a similar manner. The cells were labeled with avidin conjugates carrying multiple Eu^{3+} chelates of probe **1** with an average 1.6×10^7 probes per cell.

2.6.2. Measurement of light emission of lanthanide chelates in steady-state mode

The detection of light emission of a lanthanide chelates and their conjugates with avidin as well as of BODIPY-modified avidin was performed in a measuring cell 150 μl) in a buffer containing 10 mM Hepes pH 8.0. Water-based or deuterium oxide-based solutions were used.

3. Results

3.1. The synthesis and properties of reactive luminescent lanthanide probe 4

In our previous study [15], we found a convenient modification reaction for the $cs124CF_3$ fluorophore, which allows introduction of the crosslinking groups at N1 position. Here we performed the same reaction with parent cs124 compound in order to obtain probe **4** (Fig. 1). Similarly to corresponding trifluoro-derivative, alkylation of cs124 fluorophore by bifunctional biphenyl compound produced alkylation product at N1 with high yield (Fig. 2). Notably, alkylation proceeded almost exclusively at N-1 of the quinolone ring, while the same reactions with ethyl ester of 4-toluenesulfonic acid or with 1-iodo-3-azidopropane yielded detectable amount of O-alkylated products (15). This can be accounted for different structure of the reactive groups. Employing high molar excess of alkylating agent suppressed the formation of crosslinked quinolone adducts. After the alkylation, the remaining chloromethylene group was quantitatively converted to an azido derivative (compound **I**) by incubation with LiN_3. The later was reduced to corresponding amino-compound **II** by treatment with triphenylphosphine and ammonium hydroxide. Reactive isothiocyano-derivative **III** was obtained by subsequent incubation of **II** with thiocarbonyldiimidazole and TFA. Acylation of compound **III** with DTPA dianhydride produced final product, which was chelated with Tb^{3+} ion by addition of $TbCl_3$ to yield probe **4**.

3.2. Modification of avidin by reactive light emitting labels

As expected, incubation of various reactive fluorophores with avidin resulted in covalent attachment to the protein as judged by size-exclusion chromatography. The dependence of the number of attached fluorophore residues of probe **1**, **2**, and **4** as well as BODIPY fluorophore per avidin molecule on probes concentration is shown in Fig. 3. Since the probes are amine-reactive it is expected that they will predominantly attach to lysine residues. It can be seen that at a high concentration

24–31 out of 32 lysine residues of the protein can be modified by the probes. Attempt to attach more than 4 BODIPY residues per avidin was not successful due to precipitation of the modified protein.

3.3. Light-absorbing and light-emitting properties of the lanthanide probes and their conjugates with avidin

As seen from Fig. 4, in comparison to probe **2**, probe **4** possesses a significant absorption in the range of 240–300 nm, which is obviously due to the presence of the biphenyl chromophore. Also, modification of the cs124 moiety at N1 causes a small (6 nm) batochromic shift of the absorption in the region of 320–360 nm. Biphenyl modification only slightly affects the brightness of the chelate as compared to the brightness of previously designed probe **2** (Table 1 and Fig. 5A and B), which makes this position a convenient site for the introduction of crosslinking or other functional groups. Strong light absorption of the biphenyl group in the region 240–300 nm does not interfere with the light absorption properties of the antenna and antenna-to-lanthanide energy transfer, as biphenyl- and quinolone moieties do not form a common light-absorbing unit, being separated by methylene group. As seen from Fig. 5A, a shift in the light absorption of probe **4** results in the same shift of the fluorescence excitation spectrum. Also, the excitation spectrum of probe **4** displays a significant maximum in the region 240–300 nm where the biphenyl group absorbs the light. This is indicative for energy transfer from the excited state of the biphenyl group to the cs124 chromophore, favored by close proximity of the moieties. Heavy water caused a significant enhancement of lanthanide emission (Table 1) due to the elimination of the excitation energy dissipation by coordinated water molecule through O–H bond vibration.

Avidin represents highly stable tetrameric structure containing 32 lysine residues to which the reactive probes can be attached. Avidin binds tightly to biotin ligand producing virtually irreversible complex. This property of the protein makes it a convenient carrier for the attachment of various probes. Avidin conjugates thus obtained can be used to label biotinylated molecules of interest. It is seen (Table 1) that the attachment of Tb^{3+} luminescent chelates **2** and **4** to the protein at low concentration of the probes caused ca. 3-fold quenching comparing to emission of non-attached probes. For probe **2**, increasing the number of attached probes resulted in further progressive quenching (Fig. 5C), while for probe **4** the dependence of the cumulative fluorescent signal on the number of the crosslinked probes remained linear. Attachment of Eu^{3+}-based probe **1** also resulted in 3-fold quenching, however when the number of the conjugated probes increased, a significant super-linear luminescence enhancement was observed (Fig. 5C). This effect can be explained by enhancement of antenna-to-lanthanide energy transfer, which is supported by decrease of antenna fluorescence and simultaneous increase of lanthanide emission in the complex (Table 2). One factor that reduces the brightness of the probe could be quenching due to the contact between the antenna fluorophore and protein surface. This is supported by the superior properties of the probe **4** possessing a rigid spacer between the antenna fluorophore and the crosslinking group. This spacer could prevent the quenching by restricting the fluorophore contacts with avidin.

As expected, light emission of avidin conjugates increased in heavy water (Table 1). Thus 1.3 and 3-fold enhancement was observed for Tb^{3+} and Eu^{3+} chelates correspondingly, which is close to enhancement factors for corresponding non-attached probes [13].

As seen from Fig. 5D, attachment of more than one BODIPY fluorophore to avidin dramatically decreased the cumulative fluorescent signal due to expected FRET quenching.

3.4. Modified avidin conjugates are capable of biotin binding

Extensive modification of avidin could potentially interfere with biotin binding. To test the binding ability of the modified protein, we titrated the conjugate with biotinylated oligonucleotide carrying BHQ quencher. As seen from Fig. 6, incubation caused a dramatic decrease in brightness suggesting quenching of the modified protein through binding of the biotinylated oligonucleotide. As expected, ca. 4-fold excess of the oligo was required to achieve maximal quenching, which corresponds to saturation of all biotin binding sites.

3.5. Microscopic imaging of living cells using luminescent avidin conjugates

To image the cells, we first treated them with acylating biotin derivative, which resulted in covalent attachment of the biotin residues to the cellular surface (Fig. 7A and B). As expected, subsequent incubation with luminescent labeled avidin conjugates resulted in the attachment to the cells as judged by visual inspection under UV light. For microscopic imaging of the cells in time-gated mode we used Total Internal Reflection Fluorescence Microscopy (TIRFM) [16,17]. Total internal reflection is an optical phenomenon, which occurs when light propagating in a dense medium (such as glass) meets an interface with a less dense medium, such as water. If the light meets the interface at a small angle, some of the light passing through the interface is refracted and some is reflected back into the dense medium. At a certain angle all of the light is reflected. This angle is known as the critical angle, and its value depends on the refractive indices of the media (n_1, n_2): $\Theta_c = \sin^{-1}(n_1/n_2)$. However, some of the energy of the beam propagates a short distance (a few hundred nanometers) into the water, generating an evanescent wave. If this energy is not absorbed, it passes back into the glass. However, if a fluorophore molecule is within the evanescent wave it can absorb photons and be excited. In this way, it is possible to get fluorescence with a very low background of excitation light. We used this principle in the design of the experimental set-up for imaging of small luminescent objects (Fig. 8A). This allowed selective excitation of the surface attached objects. Repetitive laser pulses excited labeled cells and the luminescent signal collected after a short time delay allowing the decay of short-lived background fluorescence. Light emission images were acquired and accumulated using an ICCD camera. Optical and time-gated luminescent images for bacterial and mammalian cells are shown in Fig. 8B. As expected, the images were highly contrasted.

4. Discussion

This study demonstrates the fact that multiple luminescent chelates can be attached to avidin molecule to create hypersensitive affinity probes that can be coupled to various biomolecules of interest. Avidin is a convenient protein for design of such probes due to its relatively small size (4–5 nm) and large number of exposed Lys residues to which the lanthanide chelates can be attached. Using a high concentration of reactive lanthanide labels, we were able to introduce up to 30–31 luminescent residues in a single avidin molecule producing highly bright conjugates. Eu^{3+} conjugates of probe **1** displayed fortuitous additional signal enhancement apparently caused by proximation of the labels at the protein surface, which resulted in the improvement of antenna-to-lanthanide energy transfer. The nature of this effect is not quite clear. Enhanced energy transfer could arise due to scavenging of the fraction of the antenna light (that has not been transferred to the lanthanide) by another closely positioned antenna molecule,

which then transfers the absorbed energy to the chelated lanthanide. Indeed, small overlapping of the emission and absorption spectra of the antenna fluorophore of probe **1** is consistent with the suggested mechanism. Also, the excited antenna could transfer the energy to the lanthanide ion of the neighboring probe. Finally, carboxylate amino acid residues on the protein surface can be involved in the additional coordination of the lanthanide, displacing a water molecule from its coordination sphere, thereby enhancing the quantum yield of the metal emission [18]. Terbium-based multiple label constructs displayed a significant decrease of light emission comparing to the sum of equivalent number of non-attached probes, which was most likely due to the interaction of the chelate with the protein surface. Another factor of reducing the light emission could be contact quenching resulting from the approximation of the neighboring antennae-fluorophores at high labeling density. Luminescent quenching can be suppressed by the presence of a biphenyl spacer. Generally, the rigid biphenyl group can restrict the fluorophore contacts with the protein, and also prevent the contact quenching by interfering with stacking interactions of the antennae.

5. Conclusions

We obtained avidin conjugates carrying multiple lanthanide chelated with detection limit in 1–10 fM range as estimated by the detection sensitivity of single non-attached probes used for labeling. These conjugates can find wide application in biological, biophysical and biomedical studies. They can be especially useful for imaging of single molecules, biological micro objects, and body tissues as well as the development of highly sensitive assays in which the signal cannot be amplified (e.g. using PCR amplification technique).

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